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(54) Title: STRAIN OF CHLAMYDIA

(57) Abstract

The present invention relates to the discovery of a novel intracellular bacterium in hamsters with transmissible proliferative ileitis. This novel intracellular bacterium has been isolated in purified form and, on the basis of phylogenetic analysis, has been determined to possess 95 % similarity to *Chlamydia psittaci* and 98 % similarity to *Chlamydia trachomatis*. Thus, it has been discovered that the *"Campylobacter*-like organisms" long implicated in proliferative ileitis in hamsters, and likely in proliferative bowel diseases in other species as well, are not members of *Campylobacter* genus at all, but rather appear to be a novel species of *Chlamydia*.

STRAIN OF CHLAMYDIA

Description

Background of the Invention

Proliferative ileitis (transmissible ileal
5 hyperplasia; wet-tail) is a disease of hamsters
characterized by mucosal hyperplasia and by a
pyogranulomaeous inflammation of the distal ileum. The
hyperplastic phase of the disease precedes the
inflammatory phase and is characterized by replacement of
10 the normal epithelium with an immature pseudostratified
epithelium with a high mitotic index. As the disease
progresses, the villi elongate and become leaflike, and
the proliferating crypts penetrate into the underlying
supporting tissues and Peyer's patches. Secondary
15 bacterial invasion may result in abscess formation,
peritonitis, and ileal rupture. Bacteria, viral agents,
and neoplasia have all been implicated as possible
etiologies for the disease.

Light microscopic studies have revealed a
20 gram-negative, slightly curved bacillus within the
hyperplastic ileal cells of hamsters with proliferative
ileitis. An antibody reactive with this bacillus is
produced by hamsters naturally infected with the disease
and by those inoculated with homogenized ileal lesions.
25 Ultrastructurally, the organism resembles members of the
genus _Campylobacter_. For this reason, the appellation
"Campylobacterlike" has been used in the literature to
describe these mysterious bacteria, widely believed to be
yet-unidentified members of the genus _Campylobacter_.
30 Microbiologic studies have repeatedly associated
Campylobacter jejuni with both naturally occurring and
experimentally induced proliferative ileitis in hamsters.

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However, oral inoculation of hamsters with pure cultures
of <u>C. jejuni</u> has been uniformly unsuccessful in
reproducing the disease. Surgical inoculation of <u>C.
jejuni</u> into the ileum has produced diarrhea and
5   enterocolitis, but not proliferative lesions.
<u>Campylobacter hyointestinalis</u> has also been isolated from
hamsters, but attempts to reproduce proliferative ileitis
by oral inoculation of pure cultures of <u>C.
hyointestinalis</u>, as well as <u>Campylobacter mucosalis</u> and
10   <u>Campylobacter coli</u>, have also been unsuccessful.
       Proliferative bowel diseases, or enteropathies,
characterized by intraepithelial <u>Campylobacter</u>-like
bacteria are well-known in swine as well as hamsters, and
have also been reported in ferrets, sheep, rats, guinea
15   pigs, dogs, a foal, a blue fox, Moluccan rusa deer, and
rabbits.
       The etiology and pathogenesis of these proliferative
bowel diseases have been poorly understood. Efforts to
identify the intracellular bacteria in swine and hamsters
20   have been unsuccessful, and few studies have been
conducted in other species. The consistent location and
morphology of the organisms and the similar general
characteristics of the lesions in a wide variety of host
species have tended to implicate the intracellular
25   bacteria as the etiologic agent(s).
       It has been known that the disease could be produced
in pigs and hamsters by inoculation with preparations of
homogenized intestinal tissue containing intracellular
<u>Campylobacter</u>-like bacteria, and that these preparations
30   were no longer pathogenic after passage through filters
which retained bacteria. Successful treatment of enteritis

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with antibiotics in rabbits and ferrets also supported the
contention that bacteria had at least a contributing
etiologic role. However, it has not been possible to
obtain cultural isolates of the intraepithelial bacteria
5   or to reproduce the natural disease in gnotobiotic hosts.
        Porcine proliferative enteritis (swine proliferative
enteritis; porcine necrotic enteritis; porcine hemorrhagic
enteropathy; porcine intestinal adenomatosis) is a disease
which occurs naturally in swine and has been linked for
10   some time with the intracellular presence of
Campylobacter-like organisms. It is pathologically
comparable to hamster proliferative ileitis (Jonas et al.,
J. Am. Vet. Med. Assoc., 147:1102-1108), and weanling
hamsters have proven to be susceptible to the agent of
15   porcine proliferative enteritis by cross-species
transmission (McOrist et al., Veterinary Microbiology,
15:293-302 (1987)). Porcine proliferative enteritis occurs
in pigs of weaning age or older but is primarily observed
in feeder pigs (14 to 15 kg) and in gilts (bred), sows,
20   and boars. The disease is worldwide in distribution,
having been reported in the United States, United Kingdom,
Sweden, Australia, Denmark, and Taiwan.
        Gross lesions are characterized by thickening of the
wall of the ileum primarily, but also of the jejunum,
25   cecum, and colon. There are reticulation of the serosa,
hyperemia and edema of the mesentery, and enlarged
ileocecal lymph nodes. The affected intestinal segment may
contain variable amounts of fibrin, necrotic material, and
blood in the lumen. The underlying mucosa has prominent
30   mucosal folds and a granular appearance and is hyperemic.

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Occasionally, there are diffuse areas of mucosal necrosis and prominent thickening of the tunica muscularis.

Microscopically, there are diffuse proliferation of crypt epithelium, elongation of crypts, and flattening of
5 the villous surface. Variable numbers of macrophages, eosinophils, and neutrophils are present in the lamina propria. There is superficial to full-thickness mucosal necrosis, accompanied by exudation of fibrin, leukocytes, and erythrocytes into the intestinal lumen.

10 Under electron microscopy, Campylobacter-like organisms (CLO) found in conjunction with either hamster or swine enteritis are free within immature epithelial cell cytoplasm, are 2 to 3 μm long and 0.2 to 0.3 μm in diameter, and have a trilaminar, undulating cell wall. The
15 organism apparently penetrates immature crypt epithelial cells preferentially, by passing through the microvillous border of these cells. In more severely affected tissues, there is confluent multifocal to diffuse degeneration of the superficial epithelium 5 characterized by rounding,
20 vacuolation, and sloughing of epithelial cells.

While results of immunofluorescence studies have indicated that the organism within the ileal enterocytes differs from known Campylobacter species, most speculation has centered around the proposition that the organism is
25 an as yet uncultured Campylobacter species. (McOrist et al., FEBS Microbiology Letters, 69:189-194 (1990)). Despite a tremendous amount of research interest and activity in the field, the identity of this mysterious intraepithelial organism has remained shrouded in
30 speculation, due to an inability to obtain cultural

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isolates and/or reproduce the natural disease in
gnotobiotic hosts.

Summary of the Invention

      The present invention relates to the discovery of a
5 novel intracellular bacterium in hamsters with
transmissible proliferative ileitis. This novel
intracellular bacterium has been isolated in purified form
and, on the basis of phylogenetic analysis, has been
determined to possess 95% similarity to Chlamydia psittaci
10 and 98% similarity to Chlamydia trachomatis.
      Thus, it has been discovered that the
"Campylobacter-like organisms" long implicated in
proliferative ileitis in hamsters, and likely in
proliferative bowel diseases in other species as well, are
15 not members of Campylobacter genus at all, but rather
appear to be a novel species of Chlamydia. This purified
and isolated bacterium, hereinafter referred to as
Chlamydia sp. strain SFPD, was deposited at the American
Type Culture Collection (ATCC), Rockville, Maryland, on
20 August 21, 1991, and accorded Accession No. M83313.
      The present invention also relates to rapid and
effective methods for diagnosis of transmissible
proliferative ileitis. As used herein, transmissible
proliferative ileitis includes transmissible ileal
25 hyperplasia in hamsters, porcine proliferative enteritis
in swine, porcine necrotic enteritis, porcine hemorrhagic
enteropathy, and porcine intestinal adenomatosis, as well
as other of the so-called "CLO-associated" proliferative
bowel diseases in animals.

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This invention also relates to methods to identify infected swine, hamsters, ferrets, rabbits, *etc.*, especially those which are symptomatic and shedding the organism in their feces.

5   Either monoclonal or polyclonal antibodies can be utilized for such diagnostic purposes, in both feces and tissues, and can be directed against either the genus *Chlamydia* or this novel strain of *Chlamydia* in particular. Alternatively, DNA probes useful for clinical diagnosis of

10   the aforementioned proliferative bowel diseases, and characterized by a high degree of specificity and selectivity for the organism of the present invention, can be advantageously employed now that the identity and phylogenetic structure of the organism have been deduced.

15   This invention further relates to methods of transferring passive immunity to susceptible animals using antibodies that are reactive with *Chlamydia* sp. strain SFPD, and to vaccines prepared from the novel *Chlamydia* sp. strain SFPD.

20 Detailed Description of the Invention

The intracellular organism of the present invention was isolated from ileal epithelial cells of a hamster with transmissible proliferative ileitis (TPI). The procedure followed is set forth in considerable detail in Example 1,

25 but will be briefly summarized here.

The ileal epithelial cells were isolated by hyaluronidase digestion and gradient centrifugation. The ileal epithelial cell suspension was then lysed with 0.2% Triton X-100 and the intracellular organism collected by

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centrifugation following filtration of the lysed
suspension through a 0.65 µm filter.

The organism was isolated following inoculation of
70-80% confluent Intestine 407 human embryonic intestinal
5   cell (ATCC CCL 6) monolayers. Growth of the organism was
first detected at day 8 post-inoculation and was
maintained in cell culture by repeated passage.

As further described in Example 1, typical lesions of
proliferative ileitis were successfully reproduced in
10 hamsters inoculated per os with the infected cell-free
filtrate and 0.65-µm-pore-size filtrate, while littermates
given the uninfected control cellfree filtrate and
0.2-µm-pore-size filtrate remained free of disease.

The organism then was reisolated from one of the
15 0.65-µm-pore-size filtrate-inoculated hamsters which
developed proliferative ileitis. The organism was not
recovered from hamsters inoculated with uninfected control
cell filtrate or the 0.2-µm-pore-size filtrate.

Thus, the novel strain of Chlamydia appears to
20 fulfill the criteria to be considered an etiologic agent
of transmissible proliferative ileitis. The 0.2µm
pore-size filtrate did not reproduce the disease,
suggesting that the causative organism is larger than a
virus. Extensive transmission electron microscopic
25 examinations also failed to detect any viral particles or
other bacteria within the infected cells. The nearly
identical ultrastructural morphology of this novel strain
of Chlamydia within the infected cells utilized in the
examples and infected hamster ileal epithelium supports
30 the role of the bacterium in disease pathogenesis.

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Pursuant to identification of the causative agent as a novel strain of _Chlamydia_, modification of the disclosed isolation procedures to utilize antibiotics to which _Chlamydia_ are typically resistant would be advantageous.
5 Utilization of vancomycin, amphotericin B, and members of the aminoglycoside group of antibiotics (gentamicin, neomycin, streptomycin, etc.) in cell culture isolation effectively suppresses contaminating bacterial organisms while permitting _Chlamydia_ growth.

10   While a substantial body of "evidence" had evolved over the past several years suggesting that the intracellular organism observed within the intestinal cells in hamster and swine proliferative enteritis cases was a _Campylobacter_ species, heretofore, _Chlamydia_ had
15 never been implicated. In fact, respected researchers in the field had gone so far as to publish journal articles dismissing _Chlamydia_ as a potential causative agent (McOrist _et al._, _Research in Veterinary Science_, 46:27-33 (1989)). This failure to implicate, or even consider,
20 _Chlamydia_ as an agent is believed to stem, in part, from the atypical behavior exhibited by the novel strain of _Chlamydia_ described herein.

        The two known species of genus _Chlamydia_, _C. trachomatis_ and _C. psittaci_, are known to have an affinity
25 for epithelial cells of mucous membranes, and attachment and penetration appear to involve a heat-labile surface component on the infectious elementary bodies and a trypsin-sensitive receptor on the host cells.

        The elementary bodies enter by a phagocytic process,
30 creating a phagosome or inclusion in which the microcolony develops. While the novel _Chlamydia_ of the present

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invention does form inclusion bodies in culture, it tends
not to be vacuolated in the host. Thus, because this
intracellular organism was typically found free in the
cytoplasm, it was thought to be a Campylobacter rather
5 than a Chlamydia.

16S rRNA was isolated and sequenced as described in
detail in Example 2. Briefly, for 16S RNA sequencing, the
novel bacterium was isolated from infected cell culture
and media supernate by centrifugation at 800 x g to remove
10 cell debris, followed by isolation of the organism at
12,000 x g. The complete 16S rRNA sequence was determined
for the organism using a modified Sanger dideoxy chain
termination method, wherein primers complementary to
conserved regions were elongated with reverse
15 transcriptase.

Based on phylogenetic analysis, the novel strain of
intracellular bacterium was shown to be related to
Chlamydia, as discussed in Example 2. Based on this
analysis, the organism isolated in conjunction with the
20 present invention is believed to be a new strain, and
possibly a new species, of Chlamydia.

Effective diagnosis of transmissible proliferative
ileitis in hamsters, porcine proliferative enteritis, and
other proliferative bowel diseases has been hindered by
25 the time required to culture the causative organism from
fecal specimens and by the difficulty in distinguishing
the novel organism of the present invention from the
various species of Campylobacter which also inhabit the
intestines of affected species. As a result of the work
30 presented herein, development of diagnostic tools
promoting rapid and accurate assays for the presence of

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this novel strain of Chlamydia, Chlamydia sp. strain SFPD,
in biological samples taken from hamsters, swine, ferrets,
rabbits, and other species susceptible to proliferative
bowel diseases is now possible.

5   For example, as described in Example 1, polyclonal
antibodies, reactive with Chlamydia sp. strain SFPD can be
used in diagnostic immunoassys, such as ELISAs, RIAs, to
detect the presence of Chlamydia sp. strain SFPD in
biological samples which include fecal material and
10 tissue.

        Likewise, monoclonal antibodies can be generated
using the Chlamydia sp. strain SFPD, isolated by the
methods described herein, and standard techniques well
known to those skilled in the art. These monoclonal
15 antibodies can be used in diagnostic assays as described
above.

        Similarly, the isolated Chlamydia sp. strain SFPD can
be used as an antigen in an immunoassay, such as the ELISA
described in Example 1, to detect antibodies to Chlamydia
20 sp. strain SFPD found in the serum of animals infected
with the bacterium.

        Isolation of this novel strain of Chlamydia makes
possible a determination of the nucleotide sequences of
its 16S rRNA molecule, which in turn, makes possible the
25 development of nucleic acid probes to aid in its
detection.

        Nucleic acid probes are a sensitive and rapid
alternative to culture methods for the detection of
pathogens, particularly in the case of fastidious or
30 noncultivatable organisms. These probes can be either DNA
or RNA. DNA probes, for example, are now in common usage

and can be custom-designed in terms of their sizes and
specificities for a variety of prospective applications.
The sizes of these probes can range from entire plasmids
(kilobases in size) down to simple 10- to 15-base
5 synthesized oligonucleotides.

DNA probes can be tailored to bind to other DNA,
ribosomal RNA (rRNA), or messenger RNA (mRNA). A given
probe will bind to specific nucleotide sequences. A
radioactive, enzymatic, or organic label bound to the
10 probe allows it to be detected.

DNA probes specific for rRNA sequences have been used
successfully in the detection of various pathogens, and
various diagnostic kits based on DNA probes are now
available for use in clinical labs. The presence of
15 Chlamydia sp. strain SFPD in biological samples can be
detected using these nucleic acid probes. For example, a
sample of fecal material from an animal can be hybridized
to a DNA probe which is complementary to all, or a
portion, of the 16S rRNA sequence (SEQ. ID. NO: 1)
20   Five DNA probes (SEQ. ID. NOS: 3, 5, 7, 9 and 11)
which hybridize to target sequences of Chlamydia sp.
strain SFPD have been developed as described in detail in
Example 3.

Additionally, antibodies reactive with Chlamydia sp.
25 strain SFPD can be used to transfer passive immunity to
animals susceptible to the bacterium. These antibodies,
either polyclonal or monoclonal, can be formulated for
therapeutic use as a pharmaceutical composition comprising
appropriate carriers, excipients, and other
30 pharmaceutically acceptable ingredients, as is known to
those skilled in the art of pharmacology. Like other

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proteinaceous materials, the antibody preparation is
formulated as a sterile, non-pyrogenic composition for
parenteral administration. However, any pharmaceutically
acceptable route and method of administration of antibody
5 for passive transfer of immunity can be used, such as
those described in a standard reference text in this field
(e.g., Remington's Pharmaceutical Sciences).

     The isolated and purified bacterium Chlamydia sp.
strain SFPD can itself be used in a vaccine formulation to
10 be administered to susceptible animals. The Chlamydia sp.
strain SFPD can be attenuated so that, although the
bacterium remains infectious so that it retains its
ability to elicit an immune response in the animal, the
attenuated bacterium does not cause significant disease in
15 the host animal. Thus, the administration of the vaccine
formulation induces the host animal to produce antibodies
reactive against subsequent challenge with Chlamydia sp.
strain SFPD, thereby neutralizing the infecting bacterium
and preventing significant disease.

20   Attenuation can be achieved by multiple passaging of
the organism and selecting for a weakened strain.
Attenuation can also be achieved by mutation of the
bacterium using techniques known to those skilled in the
art.

25   Alternately, the vaccine formulation can be comprised
of killed, not just attenuated, Chlamydia sp. strain SFPD.
Killing can be achieved by heat treatment or lethal doses
of chemicals, such as formalin. In either case, the
bacterium comprising the vaccine formulation is capable of
30 eliciting an immune response in the host animal without
significant disease.

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The vaccine will be formulated for therapeutic use
as a pharmaceutical composition comprising appropriate
carriers, excipients, and other pharmaceutically
acceptable ingredients, as described above and as known to
5 those skilled in the art of pharmacology. The vaccine
formulation may also contain additional materials, such as
adjuvants, to increase the immunogenicity of the vaccine.
Serum titers of antibodies elicited in response to
immunization with these vaccine formulations can be
10 determined by use of the diagnostic assays described
above.

The present invention will now be illustrated by the
following examples, which further and more specifically
illustrate the invention.

15 <u>EXAMPLE I: Isolation of an intracellular bacterium from
hamsters with proliferative ileitis and reproduction of
the disease with a pure culture</u>

<u>Isolation procedures</u>

The aseptically removed ilea of three hamsters with
20 experimentally induced proliferative ileitis (21 days
post-inoculation of infected ileal homogenate) provided
the source material. The ilea were cut into 2.5-cm
sections, opened longitudinally, and repeatedly washed
with Hank's balanced salt solution (HBSS) without Ca^2+ or
25 Mg^2+ and 1 mM EDTA (HBSS-EDTA) to remove the intestinal
contents. The mucus was removed by incubating the ileal
sections in 20% (wt/vol) N-acetyl-cysteine (Sigma Chemical
Co., St. Louis, Mo.) for 45 min at 37°C with agitation.
The supernatant was decanted, and the sections were wash d

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twice in HBSS-EDTA and then three times in HBSS. The
sections were then transferred to 20 ml of hyaluronidase
(1 mg/ml) in HBSS with 5% (wt/vol) bovine serum albumin
(BSA) and incubated at 37°C for 2 hr with agitation to
5 free the epithelial cells. The resulting solutions were
successively filtered through a sterilized glass
bead-filled syringe, a 105-μm-pore-size polypropylene
mesh, and a 52-μm-pore-size nylon mesh (Spectra/Mesh;
Spectrum Medical Industries, Inc., Los Angeles, Calif.) to
10 remove the larger tissue fragments.

The crude cell suspensions were centrifuged (400 x g
for 10 min) and resuspended in HBSS twice. Following a
final centrifugation, the cell pellets were resuspended in
50 ml of Leibovitz L-15 media (Sigma) with 10% fetal calf
15 serum, 100 μg of gentamicin sulfate (Sigma) per ml, and
2/5 μg of amphotericin B (Sigma) per ml and incubated
overnight at 4°C with agitation.

The cells were separated by centrifugation (400 x g
for 10 min) and washed three times in HBSS-EDTA with 5%
20 BSA and resuspended in a minimal volume of HBSS-EDTA with
5% BSA. The cell suspensions were then layered onto
preformed (15 min at 15,000 x g and at 4°C) 30% Percoll
(Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.)
gradients in HBSS-EDTA with 5% BSA and centrifuged at 400
25 x g for 15 min. The epithelial cell bands (density 1.035
g/ml) were suspended in HBSS with 5% BSA and washed four
times in HBSS with 5% BSA followed by centrifugation (400
x g, 10 min).

The cell pellets were resuspended in HBSS with 5% BSA
30 and 0.2% Triton X-100 and incubated at 37°C for 30 min for
agitation to effect cell lysis. The suspensions were then

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centrifuged (400 x g, 10 min) to remove cell fragments, and the supernatants were filtered through stacked 20-μm-pore-size nylon (Micron Separations, Inc., Westboro, Mass.) and 0.65-μm-pore-size nitrocellulose filters

5   (Whatman Co., Clifton, N.J.). The filtrates were centrifuged at 10,000 x g for 15 min at 4°C, and the resulting bacterial pellets were washed once in HBSS, recentrifuged, and resuspended in HBSS.

Microbiologic procedures

10   Three Trypticase soy agar-5% sheep blood plates were inoculated with 250 μl of each bacterial suspension. All plates were incubated at 37°C. One plate was incubated at ambient atmosphere, one was incubated in an atmosphere consisting of approximately 6% O_2, 7% CO_2, 7% H_2, and 80% N_2

15   obtained as described previously, and the remaining plate was incubated anaerobically. The aerobic and microaerobic plates were examined daily for growth, while the anaerobic plate was examined every 3 days for growth. All plates were held for a minimum of 12 days prior to being

20   discarded.

Cell culture isolation procedures

All cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Culture media, heat-inactivated fetal calf serum, media supplements, and

25   additives were obtained from Sigma.

Intestine 407 human embryonic intestinal cells (ATCC CCL 6) and GPC-16 guinea pig colonic adenocarcinoma cells (ATCC CCL 242) were grown in Dulbecco's modified Eagle's medium with 4,500 mg of glucose per liter, 110 mg of

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sodium pyruvate per liter, 584 mg of L-glutamine per
liter, and 10% fetal calf serum (DME). HaK Syrian hamster
kidney cells were grown in Eagle's minimum essential media
with Hank's salts and 10% fetal calf serum. Vero African
5 green monkey kidney cells were grown in medium 199
containing Hank's salts and 10% fetal calf serum. All cell
lines were plated onto 75-cm^2 culture flasks (Corning
Glass Works, Corning, N.Y.) in the respective media and
incubated at 37°C in a 5% CO_2 atmosphere until
10 approximately 50% confluent. Each cell line was then
inoculated with 500 μm of each isolated bacterial
suspension and incubated at 37°C in a 6% CO_2, 6% O_2, 88% N_2
atmosphere. Cell cultures were split 2:1 by scraping on
days 4 and 9 of incubation and examined daily by phase
15 microscopy (Olympus INT; Olympus Corp., Lake Success,
N.Y.) for evidence of bacterial growth. All cell cultures
were maintained for a minimum of 14 days before negatives
were discarded.
     Cell cultures with intracellular bacterial growth
20 were continued in cell culture by scraping and splitting
through four doublings. The cell culture supernatant was
used to infect additional flasks of the same cell line to
expand the culture. Infected cells were frozen at -80°C in
the respective media with 10% dimethyl sulfoxide for
25 reference and later use.

Inocula preparation
     Four inocula were prepared for the infection studies.
The inoculum for the control group (group A) was prepared
from two 75-cm^2 flasks of confluent uninfected Intestine
30 407 cells. The cells were mechanically separated from the

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flask by scraping, concussion, and vortexing. Triton X-100 was then added to the cell media suspension to a concentration of 0.2% (vol/vol), and the suspension was incubated at 37°C with agitation for 20 min. The

5   suspension was then centrifuged (400 x g, 20 min), and the supernatant was filtered through a 20-μm-pore-size nylon filter (Micron Separations, Inc.). The resulting filtrate was centrifuged at 11,000 x g for 20 min at 4°C, and the resulting pellet was resuspended in 10 ml of DME.

10   The infected inocula were prepared in a similar manner except that a total of six 75-cm^2 flasks were processed. Following the filtration through a 20-μm-pore-size nylon filter, the infected inocula were divided into three portions. The group B inoculum was

15 processed identically to the group A control inoculum. The group C inoculum was filtered through a 0.65-μm-pore size cellulose filter (Whatman) prior to final centrifugation, and the group D inoculum was similarly filtered through a 0.2-μm-pore-size cellulose filter (Whatman) prior to final

20 centrifugation. All inocula were held at 4°C after preparation and were utilized within 3 hr of preparation.

Animals

        Four adult Syrian hamsters (Hsd;SYR) with 17-day-old litters were purchased from a commercial supplier whose

25 colony had no previous history of proliferative ileitis. The weanling hamsters were separated from their dams, sexed, weighed, ear tagged, and placed into group cages by gender. Post-inoculation, hamsters were housed in conventional polycarbonate rodent cages and provided a

30 commercial rodent diet (RMH 3000; Agway, Inc., Syracuse,

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N.Y.) and water ad libitum. Each hamster was weighed at 3-
to 5-day intervals by placing the animal in a clean
pretared container.

Animal inoculation

5   The weanling hamsters were divided into four groups
with a conscious attempt to equalize the sex and weight
distribution among the groups. Each hamster was given 7.5
mg of cimetidine (Tagamet; SK&F Lab Co., Cidra, Puerto
Rico) intraperitoneally 30 min prior to inoculation to

10   induce temporary achlorhydria. Following oral inoculation
by gavage, each hamster was given 0.2 mg of dexamethasone
(Azium; Schering Corp., Kenilworth, N.J.) intramuscularly.
        Control group A hamsters (5 male and 3 female) were
inoculated with 1 ml of a cell-free filtrate from

15   uninfected Intestine 407 cells. Group B hamsters (5 male
and 3 female) were inoculated with 1 ml of a cell-free
filtrate from infected Intestine 407 cells. Groups C and D
hamsters (4 male and 4 female each) were inoculated with
0.65-μm- and 0.2-μm-pore-size filtrates from infected

20   Intestine 407 cells.

Necropsy and tissue processing

        Hamsters were killed by an intraperitoneal injection
of 25 mg of sodium pentobarbital at day 28
postinoculation. A terminal blood sample of 2 ml was drawn

25   from the right ventricle for serum titer determination by
enzyme-linked immunosorbent essay (ELISA), and the ileum
was aseptically removed. A small section of the ileum was
fixed in McDowell-Trump fixative for light and electron
microscopy. The remainder of the ileum was either frozen

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(-80°C) for later use or further processed for organism reisolation.

Sections for light microscopy were routinely processed, embedded in paraffin, cut at 4 μm, and stained
5 with hematoxylin and eosin and with Warthin-Starr silver stains for microscopic evaluation. Light microscopic sections were coded and evaluated without knowledge of experimental group assignment.

Reisolation procedures

10   Reisolation procedures were identical to those utilized in the initial isolation with the exception that only Intestine 407 cells were inoculated for organism reisolation. The ilea of 3 hamsters from group C (0.65-μm-pore-size filtrate inoculated) and 2 hamsters
15 from group A (control cell inoculated) were independently processed for reisolation of the organism.

Transmission electron microscopy

Hamster ileal sections fixed in McDowell-Trump fixative were washed twice in 0.122 M Millonig's buffer,
20 immersed in 1.33% osmium tetroxide in 0.122 M Millonig's buffer, dehydrated in graded ethanol solutions, cleared in propylene oxide, and embedded in Epon 812. Alkaline toluidine-blue-stained thick sections were examined for selection of thin sections. Thin sections were mounted on
25 300-mesh copper grids, stained with lead citrate-uranyl acetate, and examined with a Philips 300 electron microscope.

Infected Intestine 407 cells (passage 13) for electron microscopy were removed from the culture flask by

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scraping, pelleted by centrifugation (400 x g, 10 min),
and fixed in 2.5% glutaraldehyde in Millonig's buffer. The
cells were then processed as described above except for
the necessity of pelleting the cells by centrifugation
5   between steps.

ELISA technique

        The terminal serum samples, day 28 postinfection,
were evaluated for serum titer to the organism by ELISA. A
crude whole-cell preparation was used as the antigen in
10 the ELISA procedures. Supernatant from infected Intestine
407 cell cultures (passages 25 to 32) was centrifuged at
400 x g for 10 min to remove large particulates and cells.
The supernatant was then centrifuged at 12,000 x g for 15
min at 4°C to pellet the bacteria. The pellet was washed
15 once with phosphate-buffered saline (PBS; 0.01 M, pH 7.4,
with 0.02% Na$_3$N), recentrifuged, resuspended in PBS to a
density corresponding to a McFarland standard of 2, and
refrigerated until used. Campylobacter jejuni (ATCC
29428), isolated from brucella broth, was similarly
20 processed as a control antigen.
        Ninety-six-well assay plates (E.I.A. Microtiter
Plates; Flow Laboratories McLean, Va.) were coated with a
1:64 dilution of both the isolated bacterial antigen and
C. jejuni at 100 111 per well and dried overnight at 37°C.
25 Wells were incubated with 100 111 of absolute methanol
containing 0.3% H$_2$O$_2$ for 30 min at 37°C to inactivate
endogenous peroxidase activity. The plates were then
washed twice in ELISA wash buffer (0.15 M NaCl, 0.1 M
Tris-HCl, 0.02% Tween 20 (pH 7.4)) and incubated for 1 hr
30 at room temperature with 200 μl of PBS containing 0.20%

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(vol/vol) Tween 20 and 2.5% (wt/vol) nonfat powdered milk
per well. Plates were washed twice with ELISA wash, and
100 μl of test sera diluted from 1:100 to 1:12,800 in PBS
was applied for 1 hr. at room temperature. After being
5 washed 3 times, plates were incubated for 1 hr. at room
temperature with 100 μl of peroxidase-labeled goat
anti-hamster immunoglobulin G (IgG) diluted 1:2,500 in
ELISA wash per well. Following an additional 3 washes,
bound antibody was detected by the addition of 100 μl of
10 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid)
substrate (Sigma) for 15 min., and the A_{405} was determined
with an automated ELISA reader (Titertek Multiscan; Flow
Laboratories). All assays were performed in duplicate and
were repeated if values differed by greater than 10%.
15 Absorbance values greater than 0.2 absorbance units above
that of the control (C. jejuni) were considered positive.
Pooled sera from female hamsters from a closed colony
without a history of proliferative ileitis were included
as a negative control with all tests.

20 Polyclonal antibody production
        The bacterial isolate was purified from Intestine 407
cell culture (passage 15 and 16) for antibody production.
Culture supernatant was centrifuged at 400 x g for 10 min
to remove cell debris, and the resulting supernatant was
25 filtered through a 0.65-μm-pore-size cellulose acetate
filter (Whatman). The filtrate was then centrifuged
(12,000 x g for 15 min at 4°C), and the pelleted organisms
were washed once in PBS, recentrifuged, and resuspended in
10 ml of PBS containing 4% formalin. Following overnight
30 incubation at 4°C, the organisms were again isolated by

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centrifugation, washed once in Dulbecco's PBS,
recentrifuged, and resuspended in Dulbecco's PBS to a
concentration approximating a
McFarland standard of 0.5. The organism suspension was
5 then emulsified with an equal volume of Fruend's complete
adjuvant (Sigma Chemical Co.).

Two male rabbits were utilized for antibody
production. Each rabbit was injected with a total of 0.5
ml of the adjuvant-organism mixture divided into 10 sites
10 intradermally on the back. A booster injection of 1.0 ml
of the organism in Dulbecco's PBS was given subcutaneously
at 40 days postinjection, and sera were collected 7 days
later.

The IgG fraction of the rabbit antisera was purified
15 by precipitation with ammonium sulfate (33% final
concentration) followed by ion exchange chromatography.
The resulting IgG fractions were then reprecipitated with
ammonium sulfate, isolated by centrifugation, and desalted
on a Sephadex G25 column (Pharmacia LKB Biotechnology,
20 Inc.) equilibrated and eluted with PBS.

Immunohistochemistry

Fixed ileal sections embedded in paraffin and cut to
4 μm were utilized for immunohistochemistry. Sections were
routinely deparaffinized in xylene and rehydrated through
25 decreasingly concentrated ethanol solutions. The sections
were then incubated for 1 hr in 0.03% H_2O_2 in absolute
methanol to remove endogenous peroxidase activity.
Following washing in PBS, the sections were incubated for
1 hr in a blocking solution of 0.2% (vol/vol) Tween 20 and
30 2.5% (wt/vol) nonfat dry milk in PBS. Following an

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additional wash, the rabbit anti-organism antibody was
applied at a dilution of 1:100 in PBS, and the slides were
incubated overnight at 4°C in a humidified chamber. The
slides were then washed 4 times in ELISA wash and
5　incubated for 3 hrs with affinity-purified
peroxidase-labeled goat anti-rabbit IgG (Jackson
Immunoresearch) diluted 1:500 in ELISA wash. The sections
were then washed 4 times in ELISA wash, and bound antibody
was detected with a commercial peroxidase detection system
10　for immunohistochemistry (HistoMark Black; Kirkegaard &
Perry Laboratories, Inc., Gaithersburg, Md.) by following
the manufacturer's directions. Controls to ensure
specificity included both infected and uninfected
Intestine 407 cells, preimmunization rabbit sera, and
15　organism-adsorbed immune rabbit antibody.

Results

Isolation and propagation of the organism
Inoculation of cell cultures with two of the
bacterial suspensions resulted in heavy bacterial
20　contamination and discarding of the cell cultures after 18
hr of incubation. Cell cultures inoculated with the
remaining bacterial suspension appeared to be free of
bacterial contamination, incubation was continued, and the
flasks were split (2:1) on day 4. Intracellular organisms
25　were first observed in both the Intestine 407 and GPC-16
cell lines by phase microscopy on day 8 following
inoculation. Clusters of minute organisms within the cells
were readily identified by their rapid motility. The
clusters of organisms were extremely variable in size,

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from barely discernible within the cell cytoplasm at a
4000 x magnification to large clusters completely filling
the distended cells. Occasional cells with small clusters
of organisms were observed in mitosis with the cell
5 cluster apparently transferring to one of the daughter
cells. No evidence of intracellular bacteria was observed
in either the HaK or Vero cell lines.
        The organism continued to multiply in the original
cell cultures (with additional 2:1 splittings on days 14,
10 18, and 20) through day 19 postinoculation (Intestine 407)
and day 21 postinoculation (GPC-16), when the cultures
were contaminated. The organism was readily and
continually (more than 40 passages) propagated by
infecting confluent Intestine 407 cell cultures with the
15 supernatant from infected cultures (1 ml of infected
supernatant per 25-cm^2 surface area). During the first 5
passages in cell culture, intracellular organisms could be
detected by phase microscopy within 72 hr of infection,
with the interval between infection and microscopic
20 detection decreasing in later passages. Cell culture
monolayer viability decreased from 10 days (passage 1) to
6 days by passage 5. Following the initial 5 passages in
cell culture, organisms were detected by phase microscopy
at 24 hr post cell culture infection, and monolayer
25 destruction was evident in 4 to 6 days following
infection. No changes in bacterial morphology were evident
following up to 40 passages in cell culture. Growth of the
organism appeared to be substantially greater on the
Intestine 407 cell line and passage 12 of this line was
30 utilized for inoculum preparation.

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Necropsy results

     The gastrointestinal tract of all control-inoculated
(group A) and 0.2-μm-pore-size filtrate-inoculated (group
D) hamsters appeared grossly normal at necropsy. No
5. evidence of epithelial cell proliferation or intracellular
bacteria was present on light microscopic examination of
hematoxylin- and eosin-stained or Warthin-Starry-stained
ileal sections from hamsters in either of these groups.

     In 4 (2 male and 2 female) of the 8 group B hamsters
10 (cell-free infected inoculate) and 4 (3 male and 1 female)
of the 8 group C hamsters (0.65-μm-pore-size filtrate),
the ileum was grossly thickened. Ileal wall thicknesses
varied from 2 to 5 mm in these cases. Ileal wall necrosis
and abscessation with secondary fibrinopurulent
15 peritonitis was present in 3 of these hamsters (1 from
group B and 2 from group C). Light microscopic examination
of ileal sections from hamsters with grossly evident ileal
thickening revealed immature and disorganized villus
epithelial cells with mitotic figures extending from the
20 crypt region up the entire length of the villus. Numerous
small curved bacteria were visible in the apical cytoplasm
of the epithelial cells in silver-stained ileal sections
of all hamsters with grossly evident ileal thickening. A
mixed inflammatory cell infiltrate of neutrophils and
25 macrophages was present in the lamina propria of all
hamsters with gross lesions. Mild focal epithelial cell
proliferation and intracellular bacteria were present in
an additional 2 (1 from group B and 1 from group C) of the
8 hamsters that appeared normal at gross necropsy.

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<u>Reisolation results</u>

Three of the 5 bacterial suspensions inoculated into
Intestine 407 cell cultures resulted in heavy bacterial
contamination at 18 hr of incubation, and these cultures
5 were discarded. The remaining 2 cell cultures (1 group A
and 1 group C) remained free of bacterial contamination
through day 7 of incubation when intracellular organisms
were detected by phase microscopy in the group C
(0.65-μm-pore size filtrate) inoculated cell culture.
10 Intracellular bacteria were not detected in the group A
(control) inoculated cell culture through 14 days of
incubation.

<u>Microbiologic results</u>

Of the 8 bacterial suspensions, 3 from the original
15 isolation and 5 from reisolation, only those resulting in
contamination of the cell cultures produced any growth on
Trypticase soy agar-5% sheep blood plates. <u>Pseudomonas
aeruginosa</u>, 1 to 8 colonies per 250 μl, was isolated from
all 5 contamination suspensions. No other isolates were
20 detected by any of the incubation conditions.

<u>Transmission electron microscopy results</u>

Numerous intracellular bacteria were observed within
the cytoplasm of ileal epithelial cells from hamsters
inoculated with the 0.65-μm-pore-size filtrate (group C).
25 The bacteria were free within the epithelial cell
cytoplasm. Macrophages with intracellular bacteria both
free in the cytoplasm and in phagosomes were present
adjacent to epithelial cells. The bacteria were typically
slightly curved on longitudinal sections and measured 1.4

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to 1.6 μm in length by 0.25 to 0.38 μm in width. The
bacterial cell wall appeared trilaminar and irregular. No
flagella were observed.

Examination of infected Intestine 407 cells revealed
5 the presence of bacteria morphologically identical to
those seen in the ileal cells of infected hamsters. The
bacteria in the Intestine 407 cells measured 1.4 to 1.7 μm
in length by 0.26 to 0.34 μm with a similar trilaminar
irregular cell wall. A majority of the bacteria in the
10 Intestine 407 cells were free within the cell cytoplasm,
although some were membrane bound within vacuoles.

ELISA results

Serum titers of hamsters from group A (control
inoculated) ranged from <1:100 to 1:1,600, with a median
15 titer of 1:400. The serum titers from the group D
(0.2-μm-pore size filtrate inoculated) hamsters ranged
from <1:100 to 1:800, with a median titer of 1:400. Serum
titers of hamsters from group C (0.65-μm-pore size
filtrate inoculated) ranged from 1:1,600 to >1:12,800,
20 with a median titer of 1:6,400, while the titers of the
group B hamsters ranged from 1:1,600 to >1:12,800, with a
median of 1:3,200. The serum titers of the group B and
group C hamsters were significantly (P < 0.01,
Mann-Whitney test) higher than the titers of either group
25 A or group D hamsters. The highest titers, ranging from
1:6,400 to >1:12,800, were recorded from hamsters with
grossly evident ileal thickening at necropsy. The pooled
negative control sera consistently titered at <1:100.

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Immunohistochemistry results

Organisms labeled with the polyclonal rabbit anti-organism antibody were present in the ileal sections from all 10 hamsters with histologic evidence of
5 proliferative ileitis. Organisms were primarily confined. to the apical portions of ileal epithelial cells, with few organisms being present in the lamina propria or within the lumen. The distribution of organisms corresponded to the distribution of silver-stained organisms seen on the
10 Warthin-Starry-stained sections from the same hamster. No stained organisms were present within the ileal epithelial cells from any of the 8 group A or 8 group D hamsters.

Organisms specifically labeled with the polyclonal rabbit anti-organism antibody were also present in the
15 infected Intestine 407 cells. No reactivity was present with uninfected Intestine 407 cells, and reactivity was completely eliminated by adsorption of the rabbit antisera with formalin-fixed organisms.

EXAMPLE 2:   16S rRNA sequencing of the intracellular
20 bacterium isolated in Example 1 and its identification as a novel strain of Chlamydia

16S rRNA sequencing

The complete 16S rRNA sequence was determined for the organism isolated in Example 1 using a modified Sanger
25 dideoxy chain termination method wherein primers complementary to conserved regions were elongated with reverse transcriptase. The complete sequence (SEQ ID NO:1) is shown in Table 1

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TABLE 1

UUCUGAGAAUUUGAUCUUGGUUCAGAUUGAACGCUGGCGGCGUGGAU
GAGGCAnGCAAGUCGAACG-----GAACGAUUGCUUCGGUGAUUGUUU----
AGUGGCGGAAGGGUUAGUnAUACAUAGAUnAUUUGUCCUUAACUUGGG
AAUAACGGUUGGAAACGGCCGCUAAUACCGAAUGUGGCGUAGAUUAG-G
CaUCUAAAUUACGUUAAAGAAGGGGA-UCUUCGGA-CCUUUCGGUUAAG
GGAGAGUCUAUGUGAUAUCAGCUAGUUGGUGGGGUAAAGGCCUACCA
AGGCUAUGACGUCUAGGCGGAUUGAGAGAUUGGCCGCCAACACUGGGA
CUGAGACACUGCCCAGACUCCUACGGGAGGCUGCAGUCGAGAAUCUUU
CGCAAUGGACGGAAGUCUGACGAAGCGACGCCGCGUGUGUGAUGAAGG
CUC-UAGGGUUGUAAAGCACUUUCGCUUGGGAAUAAGA-GAAGACGGUU
AAUACCCGUUGGAUUUGAGCGUACCAGGUAAAGAAGCACCGGCUnACU
CCGUGCCAGCAGCUGCGGUnAUACGGAGGGUGCUaGCGUUAAUCGGAU
UUaUUGGGCGUAAAGGGCGUGUaGGCGGAAAGGUAAGUUAGUUGUCAA
AUCUCGGGGCUCnACCCCgAnUCGGCAUCUnAAACUnUUUUUCUAGAGG
GUAGAUGGAGAAAAGGGaAUUUCaCGUGUAGCGGUGAAAUGCGUAGAUA
UGUGGAAGAACACCAGUGGCGAAGGCGCUUUUnUnAUUUAUACCUGACG
CUAAGGCGCGAAAGCAAGGGGAGCAAACAGGAUUaGAUACCCUnGUAG
UCCUUGCCGUaAACGAUGCAUACUUGAUGUGGAUGGU--CUCAAC-CCC
AUCCGUGUCGGAGCUAACGCGUUAAGUAUGCCGCCUGAGGAGUACACU
CGCAAGGGUgAAACUCAAAAGAAUUGACGGGGGCCCGCACAAGCAGUGG
AGCAUGUgGUUUaAUUCGAUGcaACGCGAAGGACCUUACCUGGGUUUGA
CAUGCA-UAUGACCGCGGCAGAAAUGUCGUUU--UCCGCAAGGA---CAU
AUGCA-CAGGUGCUGCAUGGCUGUCGUCAGCUCGUGCCGUGAGGUGUU
GGGUUAAGUCCCGCAACGAGCGCAACCCUUAUCGUUAGUUGCCAGCAC-
UUAGG--GUGGGAACUCUAACGAGACUGCCUGGGUUnACCAG-GAGGAAG
nnnAngAUGACGUCAAGUCAGCAUGGCCCUUAUGCCCAGGGCGACACACG
UGCUACAAUGGCCAGUACAGAAGGUAGCAAGAUCGCGAGAUGGAGCAA
AUCCUC-AAAGCUGGCCCCAGUUCGGAUUGUAGUCUGCAACUCGACUAC
AUGAAGUCGGAAUUGCUAGUAAUGGCGUGUCAGCCAUAACGCCGUGAA
UACGUUCCCGGGCCUUGUACACACCGCCCGUCACAUCAUGGGAGUUnG
UUUUACCUUAAGUCGUUGACUCAACCCGCAAGGGGGAGAGGCGCCCAA
GGUnAGGCUGAUG

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In the foregoing sequence, A represents the base adenine, C represents the base cytosine, G represents the base guanine, U represents the base uracil, lower-case letters (a, c, g, etc.) are believed to be their upper-case

5 equivalent although this is not known with certainty, n indicates that the identity of the nucleotide is unknown, and (-) represents a gap in the sequence as aligned with the 16S rRNA sequence of E. coli.

Similarity matrix

10   Based on phylogenetic analysis, the novel strain of intracellular bacterium was related to Chlamydia, as shown in Table 2.

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TABLE 2

C_s	C_t	C_p	C_f	H_p	H_s	P_v	
----	98.4	95.6	72.6	72.9	74.1	73.7	Chlamydia sp. strain SFPD
1.6	----	95.2	72.2	72.9	73.9	73.5	Chlamydia trachomatis
4.5	4.9	----	72.5	73.0	74.1	73.4	Chlamydia psittaci
34.1	34.7	34.2	----	85.4	76.9	76.0	Campylobacter fetus
33.7	33.7	33.5	16.2	----	75.2	75.0	Helicobacter pylori
31.7	32.0	31.8	27.6	30.1	----	92.9	Escherichia coli
32.4	32.7	32.8	29.0	30.4	7.5	----	Proteus vulgaris

The numbers above the diagonal represent percent similarity. The numbers below the diagonal are percent differences corrected for multiple base changes by the method of Jukes and Cantor (1969).

The relationship among the above species can be summarized as shown in Table 3.

TABLE 3

Chlamydia sp. strain SFPD
Chlamydia trachomatis
Chlamydia psittaci
Campylobacter fetus
Helicobacter pylori
Escherichia coli
Proteus vulgaris
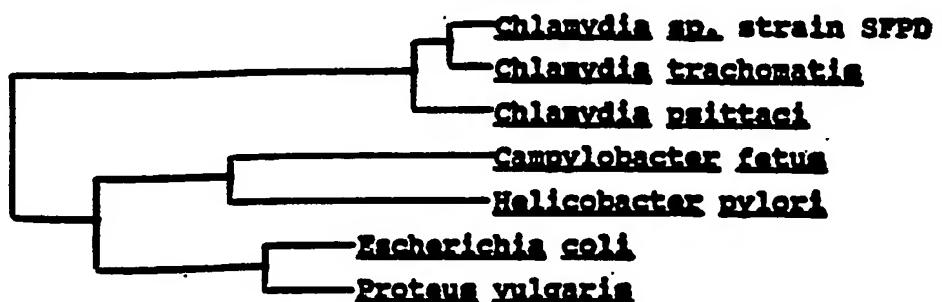
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Example 3:   Development of DNA Probes

1. Determination of oligonucleotide signatures
     Signatures are defined as those segments of the RNA
5   sequence which are unique to any given species or group of
phylogenetically-related organisms. Signatures can be
found by examination of aligned sequences, noting regions
in the desired species or higher phylogenetic group that
contain differences at sites conserved in other species.
10 Once such a sequence is found, large numbers of reference
sequences are searched (by computer) to verify the
uniqueness of the sequence. The sequence of Chlamydia sp.
strain SFPD was compared with over 350 sequences of other
bacteria. These signature regions become the target site
15 for DNA probes.

2. Probe Synthesis
     Once the desired oligonucleotide signatures are
determined for probe development, DNA that is
20 complementary to the sequence of the signature can be
synthesized using techniques well known to those skilled
in the art. For example, an ABI DNA Synthesizer can be
used to synthesize the oligonucleotides which serve as
probes.
25

3. Probe Labeling
     $^{32}PO_4$-ATP can be used as a substrate by T4
polynucleotide kinase for 5' end-labeling of DNA
oligonucleotides. Briefly, the procedure is as follows.
30 0.5 ug of oligomer (probe) and 200 uCi radiolabeled ATP
are combined and dried under vacuum. The dried pellet is

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dissolved in 0.05 ml of kinase buffer containing 11.5 U of
Polynucleotide kinase (Pharmacia). This mixture is
incubated at 37°C for 30 min. Ammonium acetate and EDTA
are then added to stop the reaction. The reaction mixture
5 (0.5 ml) is gravity loaded onto C-8 Bond-Elute columns
(Analytichem. Intern.) and is washed 3X with 1 ml of 50 mM
ammonium acetate and then once with 1 ml of 5 mM ammonium
acetate.

4. Probe specificity to RNA and DNA

10   Probes can be hybridized with rRNA samples that were
used for sequencing reactions. Once these probes have
proven to be successful using purified RNA, the probes can
be validated using pure cultures of target organisms
(Strain SFPD) and related organisms (e.g., other strains
15 of chlamydia). Probes can target 16S rRNA or 16S rRNA
regions of the genome.
    The hybridization procedure has been adapted from
Schleicher & Schuell (Keene, N.H.) and is as follows:
Approximately 2-4ug rRNA is denatured in a solution of
20 6.15 M formaldehyde in 10X SSC (NaCl/Na Citrate buffer) at
a temperature of 65°C for 15 min. The denatured rRNA is
transferred to a prewashed nitrocellulose (NC) filter in a
Slot Blot apparatus (Schleicher and Schuell). This
apparatus is a modified dot blot which allows for rapid,
25 high resolution hybridization of nucleic acids. The
denatured RNA is baked onto the filter at 80°C for 1h
under vacuum. The bake filter is placed in a seal-a-meal
plastic bag and hybridization solution containing
formamide, Salmon testis DNA, Denhardts solution, EDTA and
30 SDS is added to the bag. After 20 min incubation at the

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hybridization temperature (Th), the solution is removed
and replaced with a fresh hybridization solution
containing approximately 1 x 10⁷ cpm of labeled probe.
Hybridization of the labeled probe to the rRNA on the
5 filter is carried out at the Th for 18h. After 3 washes
at RT with 0.8X SSC (low stringency), the NC is washed 2
times at the Th with 0.8X SSC for 30 min each. Most of
the wash is removed, leaving the filter still wet. The
filter still in the baggie is read on a Betascope 603
10 (Betagen, Waltham, MA) which allows for the rapid imaging
(i.e., in less than 1 hour) and quantification of
radioactive samples.

Probe Sequences
Probe position: 63
15 Probe length: 24 -mer
Target sequence: (SEQ ID NO: 2)
Probe sequence: (SEQ ID NO: 3)

CGAACG-----GAACGAUUGCUUCGGUGA Chlamydia sp. strain SFPD
.............G.A....U.....CA. C. trachomatis L2/434
20 ......GAGUA-UUAAGAGAGC.U.C.NU Camp. fetus ss fetus
......AUGAA-GCU-UC.AGC.U.C.AG Helicobater pylori
......GUAACAG-GA.GAAGC.U.C.UC Escherichia coli
...G..GUAACAG-GAGAAAGC.U.C.UU Proteus vulgaris

In the example above, the 16S rRNA sequence of strain
25 SFPD is compared with the sequences of 6 other
microorganisms. Thus, there are 5 base differences
between Chlamydia sp. strain SFPD and C. trachomatis and 6

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base differences between Chlamydia sp. strain SFPD and C. psittaci.

    Probe position: 167
    Probe length: 24 -mer
5 Target sequence: (SEQ ID NO: 4)
    Probe sequence: (SEQ ID NO: 5)

    CGCUAAUACCGAAUGUGGCGUAGA   Chlamydia sp. strain SFPD
    ....AUAU   C. trachomatis L2/434
    .................UA.-.U   Chlamydia psittaci 6BC
10 U........UCC..ACUC.U.-CU   Camp. fetus ss fetus
    GAU.......AG..UCUC.U----   Helicobacter pylori
    A..........C..AAC.UC----   Escherichia coli
    G..........C...AC.UC----   Proteus vulgaris

    Probe position: 205
15 Probe length: 24 -mer
    Target sequence: (SEQ ID NO: 6)
    Probe sequence: (SEQ ID NO: 7)

    CUAAAUUACGUUAAAGAAGGGGA-U   Chlamydia sp. strain SFPD
    .CG.G.A..................   C. trachomatis L2/434
20 .....AC.UA...............   Chlamydia psittaci 6BC
    -GUU.AGU..GG....U-----.-   Camp. fetus ss fetus
    --------G.GG.....-----.-   Helicobacter pylori
    --------GACC.....G......C   Escherichia coli
    --------GACC....C.....U..   Proteus vulgaris

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```
     Probe position:  455
     Probe length:   24 -mer
     Target sequence:  (SEQ ID NO: 8)
     Probe sequence:   (SEQ ID NO: 9)


  5  GAAGACGGUUAAUACCCGUUGGAU    Chlamydia sp. strain SFPD
     ....G.............C.....    C. trachomatis L2/434
     ..GAUU..C.....U..AA.C...    Chlamydia psittaci 6BC
     ----------------------C    Camp. fetus ss fetus
     ----------------------     Helicobacter pylori
 10  ..GU.AA.........UU.GCUCA    Escherichia coli
     UG.U.AA.........UU.GUC.A    Proteus vulgaris


     Probe position:  591
     Probe length:   24 -mer
     Target sequence:  (SEQ ID NO: 10)
 15  Probe sequence:   (SEQ ID NO: 11)


     AGGUAAGUUAGUUGUCAAAUCUCG    Chlamydia sp. strain SFPD
     ..................GA....    C. trachomatis L2/434
     ...A.......A...U......U.    Chlamydia psittaci 6BC
     UAUC....CUU....G......AA    Camp. fetus ss fetus
 20  UA..C...C..G...G.....CUA    Helicobacter pylori
     U.U.....C..A...G.....C.C    Escherichia coli
     -AU.....C..A...G...G.C.C    Proteus vulgaris
```

Equivalents

  Those skilled in the art will recognize, or be able to
25 ascertain, using no more than routine experimentation,
  many equivalents to the specific embodiments of the
  invention described specifically herein. Such equivalents
  are intended to be encompassed in the scope of the
  f ll wing claims.

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CLAIMS

1.   A substantially pure culture of Chlamydia sp. strain
     SFPD.

2.   The substantially pure culture of Claim 1 comprising
     Chlamydia sp. strain SFPD accorded ATCC Accession
     No.M83313.

3.   A substantially pure culture of one or more of
     Chlamydia accorded ATCC Accession No. M83313,
     taxonomic equivalents thereof, and mutants or
     variants thereof.

4.   The purified and isolated organism of Claim 3
     Chlamydia sp. strain SFPD.

5.   The purified and isolated organism of Claim 4
     containing a 16S ribosomal RNA sequence encompassed
     by SEQ ID NO:1.

6.   A method for isolating Chlamydia sp. strain SFPD from
     the intestinal epithelial cells of an animal which
     comprises isolating said epithelial cells by
     hyaluronidase digestion.

7.   The method as described in claim 6 wherein said
     animal is a hamster, pig, rabbit or ferret.

8.   A method for isolating Chlamydia sp. strain SFPD
     which comprises propagating the organism in a
     reduced- xygen envir nment.

9.   A method for isolating Chlamydia sp. strain SFPD
      which comprises propagating the Chlamydia sp. strain
      SFPD on Intestine 407 human embryonic intestinal
      cells or GPC-16 guinea pig colonic adenocarcinoma
5   cells.

10. A method for reproducing transmissible proliferative
      ileitis in animals which comprises inoculating an
      animal with the substantially pure culture of Claim
      1.

10 11. A method for generating polyclonal antibodies
        reactive with Chlamydia sp. strain SFPD which
        comprises injecting a host animal with the Chlamydia
        sp. strain SFPD or cellular components thereof.

12. A polyclonal antibody reactive with Chlamydia sp.
15   strain SFDP produced by the method of Claim 11.

13. A method for generating monoclonal antibodies
      reactive with Chlamydia sp. strain SFPD which
      comprises injecting a host animal with Chlamydia sp.
      strain SFPD or cellular components thereof.

20 14. A monoclonal antibody reactive with Chlamydia sp.
        strain SFPD produced by the method of Claim 13.

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15. A method for detecting Chlamydia sp. strain SFPD in a
     biological material sample from an animal,
     comprising:
     a)   obtaining a biological sample from an animal;
5   b)   contacting the sample with an antibody reactive
          with Chlamydia sp. strain SFPD under conditions
          whereby the antibody reacts with the Chlamydia
          sp. strain SFPD and
     c)   detecting antibody-bound sample wherein antibody
10   bound to the sample is an indication of the
          presence of Chlamydia sp. strain SFPD.

16. The method of Claim 15 wherein the biological sample
     is tissue or fecal material.
15

17. The method of Claim 15 wherein the antibodies are
     labeled with a label selected from the group
     consisting fluorescein, rhodamine, and peroxidase.

20 18. The method of Claim 15 wherein the biological
     material sample is derived from hamsters, swine,
     rabbits, or ferrets.

19. The method of Claim 15 wherein the antibodies are
25   polyclonal antibodies produced by the method of Claim
     11.

20. The method of Claim 15 wherein the antibodies are
     monoclonal antibodies produced by the method of Claim
30   13.

21. A method for diagnosing transmissible proliferative
    ileitis in an animal by detecting antibody reactive
    with Chlamydia sp. strain SFPD present in the serum
    of an animal, comprising:

5   a) obtaining a serum sample from the animal;
        b) contacting the serum sample with purified and
            isolated Chlamydia sp. strain SFPD under
            conditions whereby the antibody present in the
            serum sample reacts with the Chlamydia sp. SFPD;
10   and
        c) detecting serum antibody bound to the Chlamydia
            sp. strain SFPD wherein antibody bound to the
            organism is an indication of transmissible
            proliferative ileitis.

15 22. The method of Claim 21 wherein the purified and
        isolated Chlamydia sp. strain SFPD is isolated
        according to the methods of Claims 6, 8 or 9.

    23. A nucleic acid probe for the detection of Chlamydia
        sp. strain SFPD comprising a nucleic acid which
20   hybridizes to all or a part of the sequence
        represented by SEQ ID NO:1.

    24. The nucleic acid probe of Claim 23 wherein the
        nucleic acid is DNA.

    25. The nucleic acid probe of Claim 23 as represented by
25   SEQ ID NO: 3.

    26. The nucleic acid probe of Claim 23 as represented by
        SEQ ID NO: 5.

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27.   The nucleic acid probe of Claim 23 as represented by
      SEQ ID NO: 7.

28.   The nucleic acid probe of Claim 23 as represented by
      SEQ ID NO: 9.

5 29.   The nucleic acid probe of Claim 23 as represented by
         SEQ ID NO: 11.

   30.   A method of detecting Chlamydia sp. strain SFPD
         present in a biological sample in an animal,
         comprising:
10   a)   obtaining a biological sample from an animal;
         b)   treating the sample in a manner that renders 16S
              ribosomal RNA present in the sample available
              for hybridization with a complementary nucleic
              acid sequence thereby producing a treated
15   sample;
         c)   contacting the treated sample with at least one
              probe which is a nucleic acid sequence which
              hybridizes with all or a portion of a nucleic
              acid sequence represented by SEQ ID NO:1; and
20   d)   detecting the hybridization of 16S ribosomal RNA
              from the sample with the nucleic acid probe,
              wherein hybridization is an indication of the
              presence of Chlamydia sp. strain SFPD.

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31. A method of detecting <u>Chlamydia sp.</u> strain SFPD
     present in a biological sample in an animal,
     comprising:
     a)   obtaining a biological sample from an animal;
5   b)   treating the sample in a manner that renders 16S
          ribosomal RNA regions of the genome present in
          the sample available for hybridization with a
          complementary nucleic acid sequence thereby
          producing a treated sample;
10   c)   contacting the treated sample with at least one
          probe which is a nucleic acid sequence which
          hybridizes with all or a portion of a nucleic
          acid sequence represented by SEQ ID NO:1; and
     d)   detecting the hybridization of 16S ribosomal RNA
15   regions of the genome from the sample with the
          nucleic acid probe, wherein hybridization is an
          indication of the presence of <u>Chlamydia sp.</u>
          strain SFPD.

32. The method of Claim 30 wherein the nucleic acid probe
20   is selected from the group consisting of: SEQ ID NO.:
     3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID
     NO: 11.

33. The method of Claim 30 or 31 wherein the biological
     sample is tissue or fecal material.

25 34. A method of passively transferring immunity against
     <u>Chlamydia sp.</u> strain SFPD to an animal comprising
     administering to the animal an antibody reactive with
     <u>Chlamydia sp.</u> strain SFPD.

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35. The method of Claim 33 wherein the antibody is a
     polyclonal antibody produced by the method of Claim
     11.

36. The method of Claim 33 wherein the antibody is a
5   monoclonal antibody produced by the method of Claim
     13.

37. A vaccine formulation comprising an attenuated
     Chlamydia sp. strain SFPD in which the Chlamydia sp.
     strain SFPD is infectious without causing significant
10   disease in the animal host to be vaccinated.

38. A vaccine formulation comprising killed Chlamydia sp.
     strain SFPD in which the killed Chlamydia sp. strain
     SFPD is infectious without causing significant
     disease in the animal host to be vaccinated.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/20;   C07K15/00;   C12P21/08;   G01N33/569
G01N33/577;   C12Q1/68;   A61K39/40;   A61K39/118

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12R

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X,P	BIOLOGICAL ABSTRACTS vol. 42 , 1991, Philadelphia, PA, US; abstract no. 17821, STILLS H ET AL. 'A NEW CHLAYDIA-SP STRAIN SFPD ISOLATED FROM TRANSMISSIBLE PROLIFERATIVE ILEITIS IN HAMSTERS' see abstract & MICROB ECOL HEALTH DIS vol. 4, 1991, page S99 --- -/--	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 DECEMBER 1992	8. 12. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	REMPP G.L.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	
X,P	BIOLOGICAL ABSTRACTS vol. 42 , 1991, Philadelphia, PA, US; abstract no. 17820, FOX J G ET AL. 'LOCALIZATION OF CHLAMYDIA-SP STRAIN SFPD IN CLO ASSOCIATED PROLIFERATIVE INTESTINAL TISSUE OF ANIMALS BY FA MONOCLONAL ANTIBODY AND IN-SITU DNA HYBRIDIZATION' see abstract & MICROB ECOL HEALTH DIS vol. 4, 1991, page S98 ---	1,13-16, 23,24, 30,31
Y	BIOLOGICAL ABSTRACTS vol. 67, no. 7 , 1979, Philadelphia, PA, US; abstract no. 42121, RONSHOLT T I 'INFECTION IN DANISH CATTLE' page 4181 ; see abstract & ACTA PATHOL MICROBIOL SCAND SECT B MICROBIOL vol. 86, no. 5, 1978, pages 291 - 298 ---	1
Y	INNERE MEDIZIN vol. 14, no. 2, March 1987, pages 45 - 48 W. BORNSCHEIN ET AL. 'CHLAMYDIA-TRACHOMATIS-NACHWEIS BEI ENTZÜNDLICHEN UND ULZERÖSEN ERKRANKUNGEN DES DUODENUMS UND ILEUMS' -----	1

I national application No.
PCT/US 92/07103

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 10 and 34 are directed to a method of treatment of diagnostic method practised on the human/ animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)